Production and Evaluation of Ag85B:HspX:hFcy1 Immunogenicity as a Recombinant Fc Fusion Multi-Stage Vaccine Candidate Against Mycobacterium Tuberculosis

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Research

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Abstract

Aims

Tuberculosis (TB) is one of the life-threatening infectious diseases, caused by *Mycobacterium tuberculosis* (*M.tb*). In the present study, a multi-stage *M.tb* immunodominant Fcγ1 fusion protein (Ag85B:HspX:hFcγ1) was produced and its immunogenicity as an hFcγRI targeted delivery systems for selective antigen presentation was evaluated in a mouse model.

Methods and Results

The novel Ag85B:HspX:hFcγ1 recombinant fusion protein was designed and expressed in the *Pichia pastoris* (*P. pastoris*). After affinity chromatography purification, the purity of Ag85B:HspX:hFcγ1 was confirmed by ELISA, SDS-PAGE, and Western blotting methods. The immunogenicity of the construct was evaluated by assessing interferon-γ (IFN-γ) and transforming growth factor-beta (TGF-β) in a mouse model. Co-localization results of Ag85B:HspX:hFcγ1 with hFcγRI (CD64) confirmed its function for binding with its receptor and inducing Th1 selective responses. There was a significant difference in the expression of both *IFN-γ*, ($P \leq 0.02$) and *TGF-β*, ($P = 0.05$).

Conclusions

The co-localization assay confirmed functionally the binding of the Ag85B:HspX:hFcγ1 to CD64 (FcγRI). Furthermore, *in vitro* assay showed that Ag85B:HspX:hFcγ1 can stimulate a modulated immune response in favor of anti-intracellular microbes, as IFN-γ increased, and also TGF-β as an immune-modulatory cytokine prevented the induction of hypersensitivity reactions.

Significance and Impact of Study

The combination of Ag85B as the most immunodominant *M.tb* Ag with HspX, as an Ag and adjuvant, could open a new venue for more studies for the design of multi-stage subunit vaccines for TB. Of note, an Fcγ1 fusion protein can be considered as a functional approved selective delivery vehicle for targeting antigen-presenting cells (APCs) and inducing cross-presentation.

Introduction

Tuberculosis (TB) is a re-emerging contagious disease, caused by *Mycobacterium tuberculosis* (*M.tb*). Increasing drug-resistant *M.tb* leads to death in the infected patients worldwide, and so named white death (Egedesø et al. 2020). According to the report of the World Health Organization (WHO) in 2019, nearly 10 million peoples (range, 9.0–11.1 million) were infected with TB, and 1.2 million (among HIV negative) have died worldwide (https://www.who.int/tb/publications/global_report/en/). About two billion peoples are infected with *M.tb* around the world, but only less than 10% of them appear active TB; most of the patients possess latent TB and have no clinical symptoms (Cliff et al. 2015). Based on an eligible study, the risk of infection by the active form of TB in the individuals, who co-infected

simultaneously with HIV and *M.tb* is 20–30 fold more than those infected with *M.tb* only (Purmohamad et al. 2020). Unfortunately, the emergence of the multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains to anti-mycobacterial drugs, leads to *M.tb* infection, which has become the most lethal disease (Mahajan and Dhawale 2015).

Since 1921, Bacille Calmette-Guérin (BCG) vaccine was approved by the WHO and is now used for all populations in epidemic areas. Vaccination by BCG protects infants and children efficiently against meningeal and pulmonary TB (Fatima et al. 2020). However, the protection rate of this vaccine against pulmonary TB in adults is very variable (0–80%). BCG is a live attenuated vaccine, and so should not be given to patients with AIDS (Khoshnood et al. 2018). In recent decades, studies have been performed on more effective vaccines than BCG, and some have begun the clinical trial phases. New vaccines are including recombinant BCG (rBCG), DNA vaccines, and subunit vaccines (Kaufmann et al. 2017). Recently, most studies have been conducted on subunit vaccines as new candidates for TB vaccination. In the subunit vaccine structures, one or more immunodominant antigens of microorganisms are used as a fusion construct. Moreover, compared with other new vaccines, these vaccines are more protective than the BCG vaccine and can be used as replacement or booster of the BCG vaccine (Aagaard et al. 2011).

One of the most *M.tb* immunodominant antigens is the antigen 85 (Ag85) complex. The complex includes three proteins, Ag85A (31 kDa), Ag85B (30 kDa), and Ag85C (31.5 kDa) (Babaki et al. 2017). Members of the Ag85 complex are secretory proteins, and also Ag85A and Ag85B are comprised of approximately 60% of this complex (Wiker and Harboe 1992). Ag85 complex has a different role during the intracellular pathogenesis of *M.tb*. The fibronectin-binding protein (*fbp*) encodes Ag85, and bacterium through this antigen binds to fibronectin (Forrellad et al. 2013). Furthermore, Ag85 has a mycolyl transferase activity, which is needed for attachment of mycolic acids to arabinogalactan and the formation of cord factor (Kuo et al. 2012). After the entry of *M.tb* into the cellular phagosome, the Ag85 complex inhibits the formation of phagolysosome and therefore, has a pivotal role in the intracellular pathogenesis of *M.tb* (Rens et al. 2018). Consequently, members of the Ag85 complex can promote Th1 cell response to produce interferon-gamma (IFN- γ) and interleukin-2 (IL-2) cytokines (Huygen 2014).

BCG vaccine is not able to induce Th1 response in the latent phase of TB and does not protect patients against reactivation and latent form of TB. Another *M.tb* immunodominant antigen is heat-shock protein X (HspX), which is expressed in the latent phase of the disease. This protein also named α -crystallin protein (16 kDa), which can induce a strong cellular immune response (Taylor et al. 2012).

The roles of the Fc domain of IgG1 in a fusion construct are the increase of half-life, solubility, and stability of synthetic fusion protein, and also targeted binding to the Fc gamma receptor (Fc γ R) on antigen-presenting cells (APCs). After uptake of Fc binding fusion proteins, through MHC I and II, immunity response of CD4⁺ and CD8⁺ T cell initiates and consequently leads to the elimination of microorganisms (Levin et al. 2015).

Considering BCG vaccine inefficacy, finding an efficient TB vaccine candidate is very crucial. In the present study, novel Ag85B:HspX:hFcγ1 fusion protein was designed and expressed into the *Pichia pastoris* (*P. pastoris*) system, and its immunogenicity was assessed in a mouse model.

Materials And Methods

Design of Ag85B:HspX:hFcγ1 fusion construct

The DNA fragment of Ag85B:HspX:hFcγ1 fusion was 2,048 bp in length, comprising two *M.tb* genes; *fbpB* (Ag85B), and *hspX* (HspX), bound to each other by a flexible linker, (Gly-Gly-Gly-Gly-Ser)₂. Next, fusion constructs were bound to the Fc domain (hinge-CH₂-CH₃) of human IgG1. The sequences of each gene were obtained from the NCBI GenBank database. Restriction sites of *Xho*I (5') and *Not*I (3') enzymes were included in-frame with Ag85B-(G4S)₂-HspX:hFcγ1 fusion construct (5'*Xho*I-Ag85B-(G4S)₂-HspX:hFcγ1-*Not*I3'). After final confirmation, this synthetic construct was submitted and registered in the NCBI Nucleotide database. (GenBank accession no.: **MK360906**; <https://www.ncbi.nlm.nih.gov/nuccore/MK360906.1>).

In-silico protein modeling of Ag85B:HspX:hFcγ1 fusion protein

Three-dimensional models of the Ag85B:HspX:hFcγ1 protein sequences were constructed via homology modeling. Using the basic local alignment search tool (BLAST), sequence homology searches were performed to identify the template proteins by Position-Specific Iterative-BLAST (PSI-BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>). For modeling, the *M.tb* Ag85B (Protein Data Bank [PDB] entry: 1F0N), heat shock protein 16 (PDB: 3W1Z), and human Fcγ1 (PDB entry: 1FC1) were selected as the templates of Ag85B, HspX and Fc domains, respectively. Multiple alignments were carried out on the selected sequences by ClustalX2 (protein weight matrix: BLOSUM series). Model building was performed in the program MODELLER9v20, using a model-ligand algorithm (<http://salilab.org/modeller/>) (Thompson et al. 1997). Several models at various refinement levels were generated. Three models with the lowest molecular probability density function (molpdf) score were selected for structure refinements. Finally, structure refinements and energy minimization were performed by YASARA Energy Minimization Server and Chimera 1.13 (Krieger et al. 2002; Krieger et al. 2009). The protein dimer form was created by <http://galaxy.seoklab.org/>. All models were validated, using the program ERRAT at the University of California, Los Angeles (UCLA) (UCLA, <http://nihserver.mbi.ucla.edu/saves/>) (Colovos and Yeates 1993).

The pPICZαA plasmid as a shuttle vector

Shuttle vectors can replicate into the different host cells, such as eukaryotic and prokaryotic cells. In this study, pPICZαA plasmid was used with restriction sites of *Xho*I, *Not*I, and *Sac*I and a selective marker gene, *sh ble*, as a resistant gene, indicating resistance to Zeocin antibiotic.

Codon optimizing of pPICZαA-Ag85B:HspX:hFcγ1 recombinant plasmid

Increasing the expression level needs gene optimization of fusion protein before expression in the target host. Based on the *P. pastoris* expression system, we used JCat software version 1.0 (Technical University of Braunschweig, USA) for codon optimization. Eventually, a cloned vector, pPICZαA-Ag85B:HspX:hFcy1 was made (Genray, China) along with restriction sites, and quality control of sequencing was certified.

Transformation of the cloned vector into *E. coli* Top10F'

Confirmed cloned pPICZαA plasmid was transformed into the *Escherichia coli* Top10F' (*E. coli*), as an appropriate host for replication of recombinant plasmid. The transformation was done via the chemical method, using 50 mM calcium chloride (CaCl₂). Using the Luria Bertani (LB) agar medium (HiMedia, India), containing 25 µg/mL of Zeocin (InvivoGen, USA), the growth of cloned bacteria was evaluated. After 24 h, transformed *E. coli* containing recombinant plasmid (with *sh ble* gene) were grown on LB agar surface. Subsequently, recombinant pPICZαA plasmids were extracted from bacterial cells via a plasmid extraction kit (Genet Bio, Korea).

Linearizing and cleanup of recombinant plasmids

Extracted plasmids were linearized with *SacI* enzyme (Thermo Scientific, USA). The pPICZαA plasmid has just one excision site for the *SacI* enzyme. The presence of elution buffer leads to sparking during electroporation and leads to the decrease of the electrocompetent efficacy, resulting in loss of plasmids. Therefore, the cleanup of the plasmid solution was done with the Silica Bead DNA Gel Extraction Kit (Thermo Scientific, USA), and the buffer was replaced by nuclease-free water.

Electroporation of pPICZαA–Ag85B:HspX:hFcy1 using *P. pastoris* competent cells

According to the EasySelect *Pichia* Expression Kit (Invitrogen, USA), competent cells of *P. pastoris* GS115 (Invitrogen, USA) were prepared by 1 M sorbitol. Through the crossover recombination phenomenon, linear plasmids are integrated into the *P. pastoris* chromosome. Hence, the Gene Pulser apparatus (Bio-Rad, USA) and electroporator cuvette (green cap) was used. The conditions of electroporation were as follows: 2,000 V, 200 PC (ohm), 25 C (µF), and 4.4 ms of time. Transformed yeast cells were cultured in the Yeast Extract-Peptone-Dextrose-Sorbitol (YPDS) agar medium, containing 100 µg/mL of Zeocin. After incubating plates for 2–4 days in an incubator at 29 °C, Zeocin resistant clones were grown.

Selection of the best transformant clones and colony-PCR

Based on the EasySelect *Pichia* expression manual, confirmation of integrated plasmid into the *P. pastoris* chromosome was done with universal primers, alcohol oxidase (*AOX1*) and α-factor. The length of the PCR product of the gene of interest with *AOX1* primers (5' and 3') was 2,636 bp, and with α-factor (5') and *AOX1* (3') was 2,347 bp. Also, grown clones for evaluation of the high crossover recombination were cultured on YPDS agar plates with different concentration of Zeocin, including 200, 500, 1,000, and 2,000 µg/mL of Zeocin. After 48 h, recombinant clones, growing in high levels of Zeocin (e.g., 1,000 or

2,000 µg/mL) were selected as the best recombinant clones (high-copy number integrated clones) for expression of recombinant protein in a large scale.

Optimization and large scale protein expression

In the present study, methanol concentrations, incubation times, and culture medium container were optimized. For replication and production of biomass, at first, selective clones (three clones) were cultured into 50 mL of buffered minimal glycerol medium (BMGY) in a baffled flask in a shaker incubator at 29 °C and 220 rpm, for 18–24 h, until the OD₆₀₀ of 2–6 was reached. To prevent the adhesion of secretory recombinant protein to the interior wall of the flask expanded roller bottles (Grainer, Germany) were used instead of non-siliconized baffled flasks. The roller bottle of 2,500 mL volume was filled with 250 mL of sterile buffered minimal methanol medium (BMMY). The induction of protein expression under different methanol concentrations is an important factor in the *Pichia* expression system. The primary concentration of methanol for inducing protein expression was 0.5%. The optimum methanol concentration for high-level expression of recombinant protein was 2%, but the expression was not observed in 4% of methanol. The maximum level of protein expression occurred on the 7th day of methanol induction time.

Purification of Ag85B:HspX:hFcy1 recombinant protein

According to the secretory nature of Ag85B:HspX:hFcy1 fusion protein, the supernatant of the BMMY medium was centrifuged and collected. Briefly, using 1 M sodium phosphate buffer, the pH of the supernatant was adjusted to 7 (neutral pH), and then filtrated by cellulose acetate (CA) 0.45 µm membrane filter (filtraTECH, France). Purification of protein was done by the HiTrap rProtein A Sepharose Fast Flow column (GE Healthcare, USA). At first, by using 20 mM sodium phosphate buffer pH 7, citrate elution buffer pH 3, and then again, sodium phosphate buffer, the column was regenerated. The supernatant was passed into the column (flow rate, 3 mL/min), and finally, recombinant protein was eluted by 100 mM glycine buffer pH 3. Immediately, the pH of the eluted protein was adjusted to 7 by 1 M Tris/HCl buffer pH 9. Using Vivaspinn 20 ultrafiltration spin filter (Sartorius Stedim, Germany), the protein solution was concentrated, and also glycine buffer was replaced by phosphate-buffered saline (PBS).

Analysis of Ag85B:HspX:hFcy1 protein by SDS-PAGE and Western blotting

To perform SDS-PAGE, and Western blot analyses, two gels containing 12% polyacrylamide were prepared. One gel was used for SDS-PAGE, and then protein bands were stained with Coomassie Brilliant Blue G-250 (Merck, Germany), using the Bio-Rad Mini PROTEAN II apparatus (BIO-RAD, USA). Another gel was used for Western blotting, and confirmation of recombinant protein was done by the goat anti-human IgG-HRP antibody (Santa Cruz, USA).

Co-localization of Ag85B:HspX:hFcy1 fusion protein

Binding of Ag85B:HspX:hFcy1 recombinant protein to human Fcy receptor I (FcyRI) on macrophage/monocyte as APCs, was confirmed by direct immunofluorescence assay. Shortly, 5 mL of monocytes of venous blood were separated by sterile Lympholyte-H solution (CEDARLANE, Canada). After washing by PBS and purification of monocytes, using a fixator (ethanol/acetone), monocytes were fixed on a slide in a -20 °C freezer. The slide was stained with PE anti-human CD64 (FcyRI) (BioLegend, USA), and incubated at 37 °C in a humid chamber for 1 h. The slide was washed and again incubated with Ag85B:HspX:hFcy1 protein for 1 h. Finally, the slide was washed and stained with goat anti-human IgG Fc-FITC (BioLegend, USA), and mounted with 90% glycerin and nail polish. Stained monocytes were visualized by a fluorescence microscope (Nikon Eclipse E200, Japan) with 100X magnification, and images were captured.

Preparation of Ag85B:HspX:hFcy1 subunit vaccine

The appropriate concentration of the new subunit vaccine was determined, using the bicinchoninic acid (BCA) method. Based on the universal protocol for injection of TB vaccine to mice, 50 µg/mL of recombinant protein in PBS was suitable for injection to mouse. For the best induction of cell-mediated immune (CMI) response, adjuvants dimethyl-dioctadecyl ammonium bromide (DDA) (Sigma-Aldrich, USA), and trehalose-6,6-dibehenate (TDB) (Avanti Polar Lipids; Merck, Germany) were used, in the ratio of 5 to 1, respectively ([Henriksen-Lacey et al. 2010](#)). The Ag85B:HspX:hFcy1 recombinant protein was mixed with DDA/TDB complex just 1 h before injection to the mice.

Immunization of C57BL/6 mice by Ag85B:HspX:hFcy1 subunit vaccine

Evaluation of Ag85B:HspX:hFcy1 recombinant protein immunogenicity was performed, using 6–8 weeks pathogen-free female C57BL/6 mice, purchased from the Pasteur Institute of Iran (Tehran, Iran). In brief, the mice ($n=42$) were divided into six groups. Each group includes seven mice: (a) the group receiving 100 µL of sterile PBS; (b) the group receiving 50 µL of BCG vaccine; (3) the group receiving 100 µL of Ag85B:HspX:hFcy1 (50 µg/mL) plus 100 µL of DDA/TDB; (d) the group receiving 100 µL of 50 µg/mL of Intravenous Immunoglobulin (IVIg) plus 100 µL of DDA/TDB; (e) the group receiving primed BCG, and then boosted by Ag85B:HspX:hFcy1 plus DDA/TDB; (f) the group receiving BCG and boosted by IVIg, and DDA/TDB. The groups of 1 and 2 were injected subcutaneously just one time, on the 1st day, but other groups were immunized on days 1st, 14th, 28th, and 42nd. Finally, the mice were dissected, and their spleen lymphocytes (splenocytes) were seeded in triplicate and incubated in a plate for 10–12 h at 37 °C in a CO₂ incubator. Then, cultured cells were collected and centrifuged in 1.5 mL microtubes. Supernatants were harvested, then, Wizol reagent (Wizbiosolutions, Korea) was added on cell pellets. After that mRNA was extracted, using the High Pure RNA Isolation Kit (Roche, Germany), according to the manufacturers' protocol and kept at -70 °C until further processing.

Evaluation of cytokine gene expression by qRT-PCR

First, cDNA molecules were synthesized, using a cDNA kit (Thermo Scientific) according to manufacturers' instructions. Finally, cDNA (double-stranded DNA) was amplified by quantitative real-time

PCR technique, and a Rotorgen Q-6000 real-time PCR machine (Qiagen, Germany). The expression of transforming growth factor-beta (*TGF-β*) and *IFN-γ* genes was assessed via the EvaGreen real-time PCR (Thermo Scientific, USA). The primer sequences used for *TGF-β*, *IFN-γ*, and beta-2 microglobulin (*β2M*) genes are shown in Table 1.

Table 1. Primers sequences of *IFN-γ*, *TGF-β*, and *β2M*.

Target gene	Forward (5'3')	Reverse (5'3')	Amplicon size (bp)
<i>IFN-γ</i>	TGGGTTCTCTTGGCTGTTACTG	GAGTTCCATTATCCGCTACATCTG	105
<i>TGF-β</i>	GCAAGTGGACATCAACGGGTT	CGCACGCAGCAGTTCTTCTC	192
<i>β2M</i>	CTTGTCTTTCAGCAAGGACTGG	CCACTTA ACTATCTTGGGCTGTG	127

IFN-γ. Interferon-gamma; *TGF-β*. Transforming growth factor-beta; *β2M*. Beta 2 microglobulin.

Statistical analysis

The data were analyzed, using SPSS Version 16 (SPSS, Chicago, IL, USA), and *p*<0.05 was considered statistically significant. To evaluate the gene expression levels in the mice studied group, the Kruskal-Wallis test was performed.

Results

Design of Ag85B:HspX:hFcy1 fusion construct

Firstly, the reference sequences of *fbpB*, *hspX*, and human Fc IgG1 genes were obtained from the NCBI database. The sequences were fused from N-terminus to C-terminus, with flexible linker (G₄S)₂, and the addition of *Xho*1, and *Not*I restriction sites. Sub-cloning of Ag85B:HspX:hFcy1 fusion construct was performed into the pPICZαA plasmid. The insertion site of Ag85B:HspX:hFcy1 construct was between the 5' *AOX1* and 3' *AOX1* domains in the plasmid. The Ag85B:HspX:hFcy1 fusion protein (dimer conformation) was expressed in the *P. pastoris* system. The schematic illustrations of Ag85B:HspX:hFcy1 construct and dimeric form of Ag85B:HspX:hFcy1 fusion protein were represented in Figs 1 and 2.

Molecular modeling of Ag85B:HspX:hFcy1 recombinant fusion protein

In the present study, 20 models were created using Modeler software; three models with the lowest molpdf were selected for structure refinements and the energy was minimized by YASARA Energy Minimization Server and Chimera 1.13. Among refined models, the most valid model achieved 94.55 ERRAT scores (from 0-100). Considering Fc properties in forming a dimer, our model is designated as a

dimer, which was created by <http://galaxy.seoklab.org/>. A solid ribbon model of Ag85B:HspX:hFcγ1 fusion protein is depicted in Fig. 3.

Colony-PCR of selected transformant clones

As noted, following the electroporation of the Ag85B:HspX:hFcγ1 DNA fragment into the *P. pastoris* chromosome, insertion of the construct was confirmed by *AOX1* and α-factor primers, using PCR amplification. The length of Ag85B:HspX:hFcγ1 DNA fragment was 2,048 bp. Based on the crossover recombination mechanism, using 5′*AOX1* and 3′*AOX1* primers, the primary DNA size of Ag85B:HspX:hFcγ1 was summed with 588 bp, but using 5′α-factor and 3′*AOX1* primers, the primary DNA size of Ag85B:HspX:hFcγ1 was added to 299 bp (Fig. 4).

SDS-PAGE and Western blot analyses of Ag85B:HspX:hFcγ1 fusion protein

Following purification of Ag85B:HspX:hFcγ1 fusion protein by HiTrap rProtein A Sepharose Fast Flow Column, the neutralization performed by Tris/HCl, pH 7. The protein was identified by SDS-PAGE and confirmed via Western blotting. Based on information obtained from the ExPASy database, the molecular weight of Ag85B:HspX:hFcγ1 recombinant protein was 77 kDa with a pI of 5.92, consisting of 709 amino acids (Fig. 5).

Methanol concentration for optimum induction of expression

The primary methanol concentration for induction of protein expression in *P. pastoris* system is 0.5% of BMMY volume (v/v). The maximum level of expression for all proteins is 2–2.5% (v/v) of methanol (Wang et al. 2010). Usually, expressing cells can bear the concentration of methanol up to 5%, but concentrations more than that are very toxic and inhibit the production of proteins (Shi et al. 2003). In this study, two methanol concentrations, 2% and 4%, were applied. The results of SDS-PAGE showed that optimum expression of Ag85B:HspX:hFcγ1 protein was obtained on 2% methanol concentration (Fig. 6)

APC-targeting of human monocytes by immunofluorescence assay

Binding of Ag85B:HspX:hFcγ1 recombinant fusion protein to FcγRI (CD64) of human monocyte/macrophages was confirmed, using immunofluorescence assay. Consequently, PE mouse anti-human CD64 (red) and goat anti-human IgG Fc-FITC (green) bind to CD64 and Fc domain of Ag85B:HspX:hFcγ1 recombinant fusion protein, respectively. The immunofluorescence microscope images showed that the inside and surfaces of the cells were emitted red and green signals, respectively (Fig. 7).

The *IFN-γ* and *TGF-β* expression levels

The results of the Kruskal-Wallis test showed that there is a significant difference in the *IFN-γ* expression ($P \leq 0.02$). The highest transcription level with a mean of 1.35 was shown in Ag85B:HspX:hFcγ1 + BCG group, which had been stimulated by PHA. On the other hand, the expression level with a mean of 0.03

was shown in the IVIg group, which had been stimulated by IVIg. The IVIg plays an immune modulator role (as an anti-inflammatory), so that following subcutaneous injection in mice, it causes an increase in the FcγRIIB expression on the surface of mouse macrophage cells, resulting in the inactivation of macrophages. The *IFN-γ* expression levels are shown in Fig. 8.

The results of the Kruskal-Wallis test revealed that there is a significant difference in the *TGF-β* expression ($P=0.05$). Like *IFN-γ*, the highest expression level with a mean of 0.24 was shown in the Ag85B:HspX:hFcγ1 + BCG group, which had been stimulated by PHA. On the other hand, the lowest expression level with a mean of 0.004 was shown in IVIg + BCG group, stimulated by IVIg. The *TGF-β* expression levels are shown in Fig. 9.

Discussion

Historically, regarding the lethal nature of *M.tb* and the emergence of new drug-resistant strains (e.g., MDR and XDR strains), TB treatment has encountered a serious challenge. On the other hand, the BCG vaccine has disadvantages such as low efficiency in adult pulmonary TB, (0–80%) inefficiency, latent TB, and HIV positive subjects (Farsiani et al. 2016; Kebriaei et al. 2016; Mosavat et al. 2016). Nowadays, subunit vaccines that are produced, comprising recombinant single or fusion proteins can be regarded as an appropriate alternative for the BCG vaccine (Babaki et al. 2019).

The *M.tb* infections are found in both active (10–20% of cases) and latent (80–90% of cases) forms; therefore, multistage subunit vaccines are the best candidates for TB infection, because they are created from both active and latent *M.tb* antigens (Farsiani et al. 2016; Kebriaei et al. 2016; Mosavat et al. 2016). For producing an effective subunit vaccine, there are two main strategies: firstly, using a targeted delivery system to target APCs for selective presentation to the Th0 cells, in order to induce appropriate immune responses; and secondly, utilizing an appropriate adjuvant in multi injection and as a multi booster to potentiate immune responses. The main powerful APCs such as DCs encode the FcγR1 (CD64), which can bind to the Fc fragment of human IgG1 or mouse IgG2a and induce cross-presentation phenomenon (Keler et al. 2000; Adamova et al. 2005; Rawool et al. 2008).

Although, covalently bonded protein to the Fc fragment has various advantages, including an increase of half-life, solubility, and stability of Fc-bound proteins, and increase of quality and quantity of purification (Czajkowsky et al. 2012). The main focus of our study was the selective targeting of DCs for the induction of Th1 and the production of proper cellular immunity. In addition, for inducing appropriate responses, Ag85B:HspX:hFcγ1 recombinant protein was mixed with TDB and DDA as Th1 adjuvants (Agger et al. 2008). Moreover, why Ag85 was chosen, because *M.tb* as an intracellular parasitic organism can secrete many different virulence factors such as, ESAT-6 (*EsxA*), CFP-10 (*EsxB*), PPEs and Ag85B, to intervene with the host responses and survive (Forrellad et al. 2013; Tang et al. 2014).

In our previous *M.tb*-host study in TB-positive and TB-negative patients, the gene expression assessments showed that the main virulence factor of *M.tb* was Ag85B and the complex of ESAT-6-CFP-10 that *M.tb* uses to combat host responses. On the other hand, the effective host's response to eliminate *M.tb* was a

Th1 cellular immune response, mainly against the predominant *M.tb* antigen (Ag85), implicating in the polarization of the response by the expression of transcription factor (*T-bet*), modulatory factors (IDO and FoxP3) and activation of effector macrophages, activating oxygen-dependent pathway, iNOS and proteolytic activities (Sharebiani et al. 2020). Therefore, Ag85 was used as the most virulence factor for activation and dissemination of *M.tb*, while it is the most immunodominant Ag for host immune responses to produce protective immunity.

TB is an immunopathology disease of type IV hypersensitivity and the appropriate host response is a potent Th1 along with a moderate TGF- β (Soleimanpour et al. 2015; Babaki et al. 2019). As a result, T-bet induces Th1 immune response to produce IFN- γ and IL-2, and Foxp3 induces Treg to secrete TGF- β and modulate the immune response to *M.tb* in a proper degree. The Th1 cells and their cytokines activated oxygen-dependent, proteolytic and autophagy of infected macrophages to eliminate *M.tb* (Mullen et al. 2001; Bhatt and Salgame 2007; Pitt et al. 2013). However, *M.tb*-HspX was used as the main expressed antigen in the latent phase of the disease and has also adjuvant activities (Lew et al. 2020). Using these two *M.tb* immunogens that covalently bind to Fc fragment (Ag85B:HspX:hFc γ 1) may form an immunodominant complex to induce Th1 proper immune response, which can be effective as a vaccine and also as a therapy in altering latency for activation and exacerbation of TB.

In recent years, attempts for making subunit vaccines have dedicated a large proportion of non-cellular vaccines. Several useful subunit vaccine candidates have been constructed by the use of Ag85, and some well-known are Hybrid 1 (Ag85B-ESAT-6 vaccine), HyVac4 (Ag85B-TB10.4 vaccine) and Hybrid 56 (Ag85B- ESAT-6- Rv2660) vaccines (Babaki et al. 2017). Given that, in our previous studies mouse Fc γ 2a-*M.tb* fusion recombinant proteins were used, showing acceptable results (Soleimanpour et al. 2015; Farsiani et al. 2016; Mosavat et al. 2016; Babaki et al. 2019); therefore, based on those results and more search in the literature, it seems that this complex would be more effective.

The findings of this study showed that when dimerized Fc γ fragment bound to Fc γ RI, it functionally induced Th1 and cross-presentation (Fig. 7), which consequently led to the increased IFN- γ expression (Junker et al. 2020). Focusing on a high concentration of IFN- γ alone for making a protective immune response against *M.tb* infection could not give promising results. TB is immunopathological damage to type IV hypersensitivity reactions, so that for producing protective immunity, the specific stimulator must induce precise immuno-modulatory responses.

In the present study, the presentation of TGF- β was also increased. TGF- β inhibited the excessive response of Th1 pro-inflammatory cytokines such as IFN- γ and prevented inflammatory reactions (Harris et al. 2007). Thus, Th1 imbalance can significantly promote the development and progression of TB (Reljic et al. 2009). As the present study findings showed, although the level of TGF- β increased, it was not comparable with IFN- γ and may modulate pro-inflammatory cytokines, for making such precise appropriate immuno-protective responses. Furthermore, preventing TB flare-up as an immunopathological disease requires a modulated Th1 immune response with dominated IFN- γ and TGF- β moderate secretion.

The importance of recombinant Fc-fusion proteins is inevitably dependent on their biophysical, biochemical and pharmacological characteristics, which exist in the constant region of the Fc fragment. Many medications with the FDA approved products are available for many different ranges of medical intervention (Czajkowsky et al. 2012; Soleimanpour et al. 2017). Furthermore, attempts for making appropriate vaccines against infectious diseases such as Ebola (Konduru et al. 2011), TB (Soleimanpour et al. 2015; Farsiani et al. 2016; Mosavat et al. 2016), Influenza (Loureiro et al. 2011) and SARS-CoV-2 (Ren et al. 2020) are still ongoing.

This study has some limitations; one limitation of utilizing FcγRs antigen-targeting is the potential interaction with the inhibitory FcγR (FcγR IIB) on APCs, which could lead to a tolerogenic immune response. Co-localization of yeast made Fc fusion protein and FcRs could aid in better understanding the type of immune response. Utilizing a suitable animal model such as a mouse challenge model is needed to assess the protective immune response of Ag85B:HspX:hFcγ1 against TB.

In conclusion, the co-localization experiments showed the proper binding of the present Fc fusion protein to CD64 (FcγRI) which in turn, confirms the suitable folding and functionally active protein for inducing Th1 responses. Furthermore, consistent with this finding, *in vitro* assay showed that Ag85B:HspX:hFcγ1 was able to stimulate a modulated immune response in favor of anti-intracellular microbes, as IFN-γ was increased, as well as TGF-β as an immune-modulatory cytokine, which can prevent the induction of hypersensitivity reactions. From our previous studies focusing on making immunogene *M.tb* fusion, taken together, it seems to us that Ag85B as the most immunodominant Ag in binding to HspX as an *M.tb* Ag and active adjuvant may provide a new venue for more attempts in making subunit multi-stage vaccine for TB. Of note, Fcγ1 fusion protein can be considered as a functional approved selective delivery vehicle for targeting APCs and inducing cross-presentation.

Declarations

ETHICS APPROVAL

The animal experiments were reviewed, and approved by the Institutional Animal Care and Use Committee of Mashhad University of Medical Sciences (**IR.MUMS.REC. 951414**) and has been performed according to National Institutes of Health guideline (**NIH publication No. 85-23, revised 1985**). During the experiments, the vaccinated mice were monitored every day.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated and/or analyzed during the current study are included in this paper and available from the corresponding author (S.A.R. Rezaee) upon reasonable request.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest regarding this manuscript.

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AUTHORS' CONTRIBUTIONS

Doing experiments and manuscript drafting: MK, AM, SS and HF; *In Silico* protein modeling: AA; Research advisors: SS, HF, MS and AM; Research director, Conception and design of the study, Data analysis: SAR and KG. All authors have read and approved the final manuscript.

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Figures

Figure 1

Schematic illustration of Ag85B:HspX:hFcγ1 fusion construct. Primary design of Fc fusion recombinant protein with XhoI and NotI enzymes restriction sites and position of its subcloning in the pPICZαA plasmid. Ag85: antigen 85B; HspX: heat shock protein X; hFcγ1: human Fc gamma receptor 1; Linker: (Gly4Ser)2.

Figure 2

Dimeric form of Ag85B:Hsp:hFcγ1 fusion protein. Ag85: antigen 85B; HspX: heat shock protein X; hFcγ1: human Fc gamma receptor 1; Linker: (Gly4Ser)2.

Figure 3

Molecular modeling of Ag85B:HspX:hFcγ1 fusion protein. Ag85B:HspX:hFcγ1 recombinant protein was modelled by MODELLER9v20. Energy minimization performed by YASARA Energy Minimization Server and Chimera 1.13 and validated by programs ERRAT in which the best final model achieved 94.55 score. The protein dimer form created by <http://galaxy.seoklab.org/>. Graph was created using Accelrys DS Visualizer v2.1. Ag85: antigen 85B; HspX: heat shock protein X; hFcγ1: human Fc gamma receptor 1.

Figure 4

Confirmation of cloned DNA fragment of Ag85B:HspX:hFcy1 fusion by PCR. Lane M, 100 bp plus DNA marker; Lane 1, *P. pastoris* housekeeping gene (~ 2.2 kp) as internal control by use of 5'AOX1 and 3'AOX1 primers; Lane 2, our interest DNA fragment plus 588 ($2,048 + 588 = 2,636$ bp) and internal control (~ 2.2 kp) by use of 5'AOX1 and 3'AOX1 primers; Lane 3, Ag85B:HspX:hFcy1 DNA fragment plus 299 ($2,048 + 299 = 2,347$ bp) by use of 5' α -factor and 3'AOX1 primers. Ag85: antigen 85B; HspX: heat shock protein X; hFcy1: human Fc gamma receptor 1; AOX1: alcohol oxidase.

Figure 5

Identification and confirmation of Ag85B:HspX:hFcγ1 protein by SDS-PAGE, and Western blotting. (A) 12% SDS-PAGE analysis of the purified protein Ag85B:HspX:hFcγ1: Lane M, protein marker (kDa); Lane 1, Ag85B:HspX:hFcγ1 recombinant protein. (B) Western blotting analysis of the purified protein: Lane 2, confirmation of recombinant protein by the goat anti-human IgG-HRP antibody. Ag85: antigen 85B; HspX: heat shock protein X; hFcγ1: human Fc gamma receptor 1.

Figure 6

Optimum and non-toxic concentration of methanol for Ag85B:HspX:hFcγ1 expression. Lane M, protein marker (kDa); Lane 1, usage of 4% methanol concentration in BMMY medium and very low level expression of protein, so that no bands were seen on SDS-PAGE gel; Lane 2, usage of 2% methanol concentration with maximum level expression of protein in BMMY medium. Ag85: antigen 85B; HspX: heat shock protein X; hFcγ1: human Fc gamma receptor 1.

Figure 7

Co-localization of Ag85B:HspX:hFcy1 protein by immunofluorescence assay. (A) Human monocytes were stained only by PE mouse anti-human CD64 (red), and were seen with red signal. (B) Human monocytes which bonded to Ag85B:HspX:hFcy1 protein were stained by both immunofluorescence colors, PE mouse anti-human CD64 (red) and goat anti-human IgG Fc-FITC (green); insides, and surfaces of the cells were seen with red and green signal, respectively. Ag85: antigen 85B; HspX: heat shock protein X; hFcy1: human Fc gamma receptor 1.

Figure 8

The IFN- γ expression levels index in the mice studied groups.- The highest expression level was observed in Ag85B:HspX:hFc γ 1 + BCG group (green bar) and vs, the lowest expression level was related to IVIg group (red bar). The words have been separated by a dash; word(s) in the left of dash: injected substance(s), and word(s) in the right of dash: stimulating substance(s). Ag85: antigen 85B; HspX: heat shock protein X; hFc γ 1: human Fc gamma receptor 1; IVIg: Intravenous Immunoglobulin.

Figure 9

The TGF- β expression levels index in the mice studied groups.- The highest expression level was observed in Ag85B:HspX:hFc γ 1 + BCG group (green bar) and vs, the lowest expression level is related to IVIg + BCG group (blue bar). The words have been separated by a dash; word(s) in the left of dash: injected substance(s), and word(s) in the right of dash: stimulating substance(s). Ag85: antigen 85B; HspX: heat shock protein X; hFc γ 1: human Fc gamma receptor 1; IVIg: Intravenous Immunoglobulin.